High throughput cell-based screening of TNF4 receptor inhibitors by homogeneous measurement of p38 MAP kinase phosphorylation

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INTRODUCTION

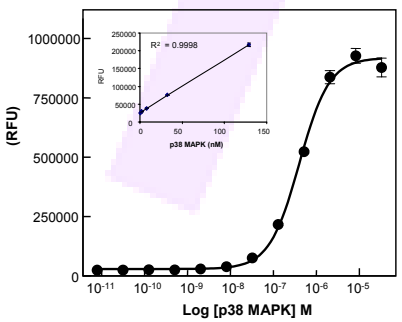
Tumour necrosis factor-α (TNFα) is a potent pro-inflammatory cytokine implicated in several autoinflammatory diseases. For the therapies that have already been developed for the treatment of TNFα related diseases, subgroups of patients may experience significant side effects, indicating a need for further drug classes that target TNFα receptor signaling. Here we present a novel method for functional screening of TNFα Receptor 1 in a cell based format. Endogenous cellular p38 MAPK phosphorylation is measured as a functional readout of TNFα receptor 1 engagement in U937 cells.

The assay utilizes the *SureFire*™ p38 MAPK assay kit (TGR BioSciences), an AlphaScreen® (PerkinElmer®) based homogeneous detection technology. We show that TNFα stimulation of p38 MAPK is markedly reduced by pretreatment of cells with soluble TNFα Receptor 1, or with antibodies against either TNFα or the TNFα Receptor 1, mirroring the currently available therapies that specifically target TNFα action. In addition, the assay is insensitive to DMSO concentrations of up to 1%, allowing screening of large drug-like compound libraries for anti-TNFα activity. Screening in a cell based format allows for both functional assessment of inhibitors in a cellular environment as well as measurement of potency in the same assay.

RESULTS

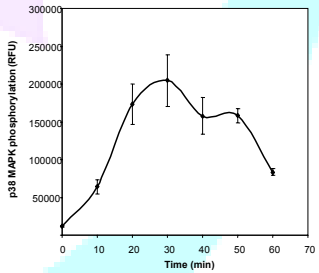
Assay Sensitivity

Recombinant p38 MAPK was used to assess the minimum detectable amount of phosphorylated p38 MAPK using the *SureFire*™ assay. Under these conditions approximately 5 femtomole/well p38 MAPK could be detected per well (approx 1nM solution), and the assay was linear between 2-200 nM, concentrations routinely expected in cell lysates.



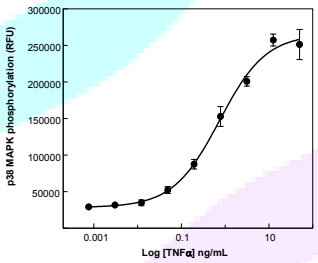
Timecourse of p38 MAPK activation in U937 cells

U937 cells (5μL) were plated into 384 well plates at a density of 10^7 cells/mL. The cells were stimulated with 5 ng/mL TNFα (5μL) for varying lengths of time prior to lysis. The cell lysates were analysed for phosphorylated p38 MAPK using the *SureFire*™ assay. The results suggest the phosphorylation maximum occurs between 20-30 min after stimulation. The mean of 3 independent experiments +/- standard deviation are plotted.



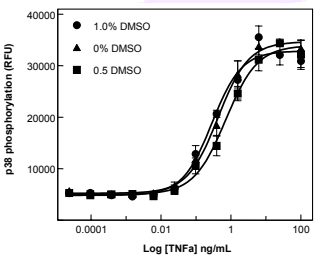
Specific p38 MAPK activation in response to TNFα in U937 cells

To ensure that p38 MAPK phosphorylation was a specific response to TNFα stimulation, the cells were stimulated with a range of TNFα concentrations, and lysed. The lysates were examined for p38 MAPK phsophorylation using the *SureFire*™ assay. The levels of phosphorylated p38 MAPK that were detected increased in a dose-responsive manner with the amount of TNFα stimulation, a response which was saturable at approximately 10 ng/mL TNFα.



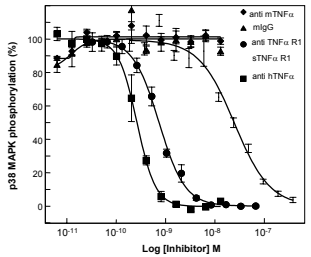
Effect of DMSO on p38 MAPK activation in U937 cells

Small molecule libraries used in HTS programs are generally stored in DMSO, a compound that can have deleterious effects on certain cells. TNFα was diluted in media containing up to 1% DMSO without any significant effect on agonist mediated p38 MAPK phosphorylation, both in terms of net signal, and EC_{50} values.



Ranking TNFα signalling inhibitors using p38 MAPK phosphorylation

Either the soluble TNFα R1, or antibodies recognising hTNFα, mTNFα of TNFα R1 were preincubated with U937 cells for 1 h prior to stimulation with TNFα. Cell lysates were subsequently analysed for phosphorylated p38 MAPK as an indicator of receptor signalling. The results suggest antibodies against either TNFα or TNFα R1 were far more potent inhibitors of receptor signalling than the soluble receptor. As expected, neither the antibody rasied against mTNFα, nor control mouse IgG, had any effect on receptor signalling.



Assay variability

Various parameters were used to measure the variability of the assay. When using recombinant phosphorylated TNFα, variation was very low, with Z' scores usually over 0.9. When measuring phosphorylated p38 MAPK in U937 cells, the variation was often higher, with CV's across the plates of activated cells often in the range of 5-10%, and of unactivated cells in the range of 10-15%. These cell based assays usually gave Z'scores in the range of 0.5 - 0.65. Because the variation in the *SureFire*™ assay itself was low, as evidenced by using recombinant material, most of the error was likely attributable to the cell system itself. However, this variability is well within the range acceptable in HTS screening programs (Z'>0.5).

CONCLUSIONS

TNFα, a potent pro-inflammatory cytokine, is implicated in several autoinflammatory diseases. Currently the therapies that have been developed may result in significant side effects, indicating a need for further drugs that target TNFα signaling. We have presented a novel method for functional screening of TNFα Receptor 1 signaling in a cell based format, based on specific TNFα-induced phosphorylation of p38 MAPK in U937 cells. Endogenous cellular p38 MAPK phosphorylation is measured as a specific functional readout of TNFα receptor 1 engagement in U937 cells. Phosphorylated p38 MAPK is measured using the *SureFire*™ p38 MAPK assay kit (TGR BioSciences), an AlphaScreen® (PerkinElmer®) based homogeneous detection technology. The assay has acceptable variability and is compatible with DMSO concentrations of up to 1%, allowing screening of large drug-like compound libraries for anti-TNFα signalling activity. Although not tested here, this assay is likely to be equally applicable in other cells that carry the TNFα Receptor 1.

Contact Information

The *SureFire*™ Cellular Kinase assay kits are formulated for the HTS marketplace for screening large sample numbers and robotic operation, as well as the research laboratory. Further information about kits, prices and protocols can be obtained from the TGR BioSciences Pty Ltd and PerkinElmer Customer Service centres:

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